Advanced polymers for DNA separation

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Recent research to improve matrices for DNA separation has resulted in the development of advanced polymers for use in capillary electrophoresis and, more generally, for electrophoresis in microchannels. To date, the most commonly used matrix is linear polyacrylamide (LPA). Unfortunately, the high-molecular weight LPA solutions required for achieving good resolution lead to very viscous solutions. Moreover, the coating ability of LPA is very poor. For these reasons, many research groups have developed low-viscosity matrices, which make microchannel filling easier, and self-coating matrices, which are able to reduce efficiently the electro-osmotic flow and the interaction of DNA with the capillary wall. To this purpose, thermo-adjustable viscosity polymers represent a very clever and interesting class of matrices.

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Abbreviations

AM	acrylamide
B	polyoxybutylene
DEA	diethylacrylamide
ds	double-stranded
E	polyoxyethylene
IPN	transient interpenetrating network
LCST	lower critical solution temperature
LPA	linear polyacrylamide
P	polyoxypropylene
PAM	polyacrylamide
PDMA	*N,N*-polydimethylacrylamide
PNIPAM	poly(*N*-isopropylacrylamide)
PVP	polyvinylpyrrolidone
PHEA	poly-*N*-hydroxyacrylamide
ss	single-stranded

Introduction

Capillary electrophoresis is rapidly becoming the reference method for DNA analysis. The most remarkable achievement of this technique has been the sequencing of the human genome, which was performed twice as fast as anticipated thanks to the development of fully automated capillary array electrophoresis sequencers [1].

The choice of the separation matrix dramatically influences separation performance and, therefore, its possible applications. Concerning DNA sequencing, LPA (linear polyacrylamide) is currently the best performing matrix in terms of read length and separation time [2–8]. At this time, optimal results are obtained with a 2.5% w/v mixture (2% w/v LPA 1.7×10^7 Da plus 0.5% w/v LPA 2.7×10^5 Da) and ≅1300 bases have been sequenced in 2 h with 98.5% accuracy for a single-stranded (ss) DNA M13 template [6]. These performances, however, have required several years of effort and optimisation. In particular, such results can only be obtained with polymers of extremely high molecular weight (typically higher than 10^7 Da), which are obtained by emulsion polymerisation [9]. Commercial solutions based on this polymer, such as *CEQ separation gel I*™ (Beckman Coulter [10]) or *LongRead*® *matrix* (Molecular Dynamics [11]), lead in routine applications to a read length in the order of 700 bases in less than 2 h: this is much less than obtained with the ultra-high molecular weight polymers, but these are the best commercial matrices in this respect.

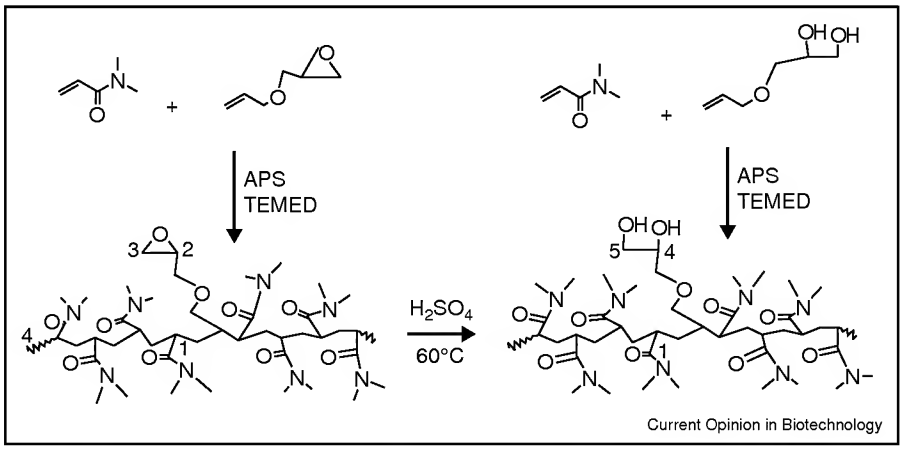
LPA has two major disadvantages, however: it has very high viscosity owing to its high molecular weight and no coating ability. Even if LPA is a shear-responsive polymer, viscosity still remains very high. For example, the zero-shear viscosity of a 2% w/v LPA solution (9×10^6 Da) is equal to 260 000 mPas compared with 27 000 mPas at a shear rate of 1.32 s^{-1}. Therefore, capillary filling becomes difficult and requires a high loading pressure (7×10^6 Pa or more). Because LPA has a poor coating ability, it has very poor electro-osmotic flow regulation [12] and capillaries need to be coated before use. (Electro-osmotic flow is a motion of the fluid induced by the counterions present in the Debye layer close to the charged capillary wall. In practice, a dispersion is observed that probably arises from unavoidable non-uniformity of the capillary surface potential.) This is why a lot of work has been devoted to developing low-viscosity and/or self-coating matrices [13–15,16•].

In this review, we focus on the most recent advances made in the development of matrices for DNA separation in capillaries and more generally in microchannels. First we discuss self-coating matrices, and then thermo-adjustable-viscosity polymers.

Self-coating matrices

Permanently coated capillaries are rather expensive to produce. Because the coating polymer can be hydrolysed or fouled with time, such capillaries have a moderate lifetime. Moreover, they cannot be regenerated when

Figure 1



Schematic representation of the synthetic pathway of hydroxylated copolymers. The left-hand side of the figure shows the synthesis of EPDMA, which is then hydrolysed with H_2SO_4 into HYDRO-PDMA. On the right-hand side the synthesis of OH-PDMA is shown. APS, ammonium persulfate; TEMED, *N,N,N,N*-tetramethylethylenediamine. (Reprinted from [20] with permission.)

resolution degrades [17]. Therefore, a lot of work has been devoted to developing self-coating matrices. Such matrices have a sufficient affinity with the capillary wall to adsorb on to it and prevent electro-osmosis or adsorption of the substances to be analysed. They can be operated in non-coated fused silica capillaries, and the capillary can be regenerated (to some extent) by acidic and alkaline treatments [17,18••]. Recently, a very interesting study on the critical factors (e.g hydrophobicity of the polymer and contour length) that control the formation of dynamic wall coatings has been carried out [19].

Poly(dimethylacrylamide) and its derivatives

Poly(dimethylacrylamide) (PDMA) is a low-viscosity matrix currently used in capillary electrophoresis. For example, the viscosity of a 6.5% w/v PDMA solution (98 000 Da) in 100 mM TAPS (pH 8), 8 M urea at 30°C is ≅75 cP. Moreover, PDMA has the advantage of being self-coating [12,20]. The best performance was obtained using a 2.5% w/v solution of high molecular mass PDMA (5.2×10^6 Da) [20]. Song *et al.* [21] sequenced 800 bases in 96 min with a final resolution of 0.5, and 1000 bases with a final resolution of 0.3 (≅limit of resolution for the best basecallers, that is, software that determines the sequence from the raw data). This group also investigated the influence of the addition of a small amount of montmorillonite clay ($2.5–5.0 \times 10^{-5}$ g/mL) into a 5% w/v PDMA solution with a low molecular weight (10^5 Da). This turned out to be a promising method to improve PDMA performance for double-stranded (ds) DNA as well as for ssDNA separation. This can be explained by the fact that the clay sheet works as a 'cross-linking' plate that increases the apparent molecular weight of the matrix and thus its sieving properties [22].

However, PDMA performances remain low compared with LPA. This may be in part due to its higher hydrophobicity and thus to specific DNA–matrix interactions [23•]. It has been shown that the performance of PDMA, and in particular the efficiency parameter, may be improved through co-polymerization of DMA with hydrophilic monomers such as 3-allyloxy-1,2-propanediol (OH-PDMA) and allyl glycidyl ether (EPDMA) [24]. Hydrolysis of EPDMA [23•] allows the formation of HYDRO-PDMA, which has the same formula as OH-PDMA (Figure 1). EPDMA has previously been studied as a coating polymer by Chiari *et al.* [25]. However, only HYDRO-PDMA gives better results than PDMA of similar molecular weight and only for the separation of DNA fragments larger than 400 bases. Given that they have similar molecular mass and co-monomer composition, the strong differences observed between these two polymers (OH-PDMA and HYDRO-PDMA) suggest that they have different conformations.

Combining acrylamide and *N,N*-dimethylacrylamide

Copolymers combining good sieving properties of LPA with the self-coating ability of PDMA have also been developed. A random copolymer with a 3:1 acrylamide (AM) to DMA ratio shows better performance than LPA of similar molecular weight (2.2×10^6 Da). A 2.5% w/v solution of this 3:1 AM:DMA copolymer allows the sequencing of 963 bases in 80 min, with a final resolution of 0.3 [26•]. Good performance was also obtained with a block copolymer of rather low viscosity (200 cP, 3% w/v in water). This matrix consists of a hydrosoluble backbone of polyacrylamide (PAM) grafted with short-chain PDMA (42 500 Da, 11.6% w/v) that can adsorb onto the capillary walls. Using a 3% w/v solution of this block copolymer (1.74×10^6 Da), 900 bases were sequenced in 110 min

[27•]. It is also possible to use PDMA as a dynamic coating additive, when used in small amounts (to avoid polymer incompatibility). Indeed, a mixture of 2.5% w/v PAM (2.2×10^6 Da) and 0.2% w/v PDMA (8000 Da) has been developed and has shown a higher efficiency in electrophoretic separation when compared with PAM alone [28].

Transient interpenetrating network

A new concept, called interpenetrating network (IPN), was first studied with polyvinylpyrrolidone (PVP) and PAM on dsDNA separation [29]. This name was given by analogy with the IPN prepared by the cross-linking polymerization of two distinct networks, although in the present case the 'network' is only a dynamic one and consists of polymerizing PAM in a PVP solution. PVP has a very low viscosity (27 cP at 4.5% w/v) and excellent self-coating properties. Sequencing of M13mp18 showed good resolution up to 500 bases in a solution of high molecular weight fraction extracted from a commercially available PVP [30]. To create a PAM and PVP IPN, PAM is polymerised in a PVP solution and, thus, the incompatibility of these two polymers is suppressed. Good results were obtained for dsDNA separation (Pbr322/*Hae*III) with a solution of 2% w/v PVP (10^6 Da) plus 1% w/v PAM (4×10^5 Da). All fragments were separated within 6.5 min. Such resolutions have been reached with a higher concentration (6% w/v) of 4.10^5 Da PAM and consequently at a rather high viscosity. However, these resolutions could not be obtained with a 10^6 Da PVP solution, even at a concentration as high as 15% w/v. An IPN of PAM and PVP was shown to be an interesting self-coating and low-viscosity matrix.

More recently, the same concept was used by replacing PAM with PDMA. First, PDMA and PVP homopolymers were synthesised and used as a self-coating matrix for Pbr322/*Hae*III DNA separation [31]. At a concentration below 4% w/v, PVP showed very poor separation of the DNA fragments, but a real improvement could be observed by increasing the concentration to 6% and 8% w/v. If a 4% w/v PDMA solution is used, separation is better than with PVP at similar concentrations, but DNA fragments above 400 bp could not be clearly separated. The poor performance of both homopolymers, in particular concerning the larger DNA fragments, is probably because of a large pore size. No real improvement was observed when using a solution of 4% w/v PDMA and 4% w/v PVP. This may be attributed to the fact that when simply mixing solutions a microphase separation can occur because of the polymers incompatibility. Then, even if the pore size is decreased, DNA can migrate through interfaces between different domains. However, when an IPN was created by polymerising DMA directly in PVP solution (10^6 Da), separation performances were strongly improved [31]. The higher sieving ability of the IPN is attributed to a smaller pore size compared with homopolymers and to an increase in the number of entanglements resulting in a stabilised network. Resolution is strongly influenced by PVP and PDMA concentrations. In optimal conditions (4% w/v PDMA and 4% w/v PVP), the 22 fragments of Pbr322/*Hae*III DNA, even the doublet of 123/124 bp, which differs by only one base pair, was separated in 15 min [31]. Up to now, no sequencing experiments have been done with these matrices.

Poly-*N*-hydroxyethylacrylamide (polyduramide™)

The *N*-hydroxyethylacrylamide monomer was found to be more hydrophilic than acrylamide and *N,N*-dimethylacrylamide [32]. So, by using polymers synthesised from this monomer (i.e. poly-*N*-hydroxyethylacrylamide [PHEA]), DNA–matrix interactions might be reduced and separation performance might be improved if compared with the interactions and separation performance of LPA. However, at this time, no data are available to compare PHEA performance with that of LPA in the separation of DNA fragments of similar molecular weight.

PHEA exhibits good coating ability and reduced electro-osmotic flow, which are similar to the properties of PDMA. Indeed, PHEA has been used for 600 h of electrophoresis under sequencing conditions with a stable and efficiently reduced electro-osmotic flow. The use of a 6% w/v PHEA solution (5.2×10^6 Da) in a bare fused silica capillary allows the sequencing of 620 bases in about 3 h, at 98.5% accuracy [32].

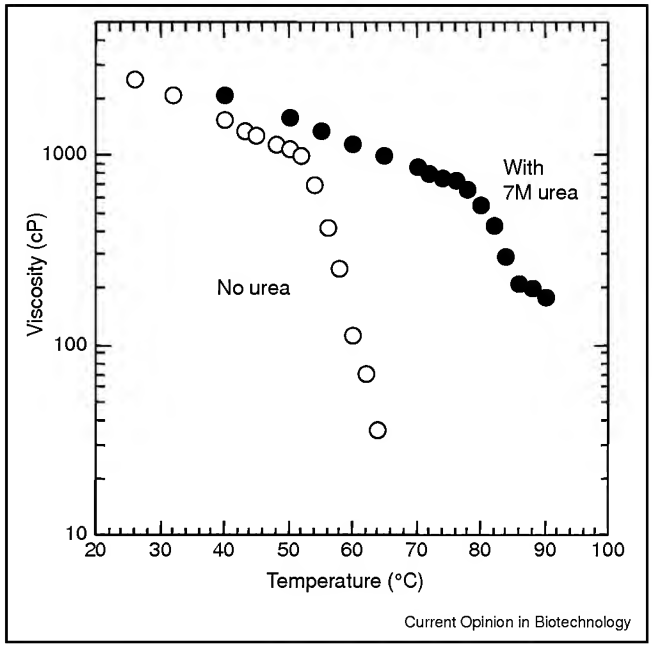
Thermoadjustable-viscosity polymers

As it has been mentioned, high viscosity is a real disadvantage for separation in microchannels. Finding low-viscosity matrices with high sieving performance is still a challenge and adjustable-viscosity polymers represent an interesting alternative. For more details, this class of matrices has been recently reviewed in [33•].

Thermothinning poly(DMA-*S*-DEA) copolymers

This random copolymer, made of *N,N*-dimethylacrylamide (DMA) and diethylacrylamide (DEA), presents interesting thermoreversible properties. At room temperature it behaves as a typical entangled polymer solution. Then, above a specific temperature called LCST (lower critical solution temperature) a microphase separation occurs and viscosity decreases quite rapidly as shown in Figure 2. More precisely, heating makes hydrogen bonds between the polymer chains and solvent get weaker and weaker. Hence, polymer chain solvation is broken and intrapolymer hydrophobic interactions increase. As a result, polymer chains collapse in coil volume. This class of copolymers was first used as a matrix for DNA separation by Sassi *et al.* [34]. Capillary filling is facilitated at elevated temperature where viscosity is relatively low. Indeed, for a 6% w/v copolymer solution (70% DEA and 30% DMA) the LSCT is 56°C and viscosity $\cong$ 500 cP at 25°C compared with less than 10 cP at 70°C. Separation is made at room temperature,

Figure 2



Rheological characterisation of thermoresponsive 53% DEA/47% DMA copolymer solutions as a function of temperature. The copolymer is at a concentration of 7.35% w/v in 50 mM Tris/50 mM TAP/2 mM EDTA. Filled circles, with urea; open circles, without urea. For both samples, the shear rate was 11.6 s^{-1}.

through the entangled network formed by the polymer solution. In this first study, the use of a 6% w/v copolymer solution made of 70% DEA and 30% DMA allowed the sequencing of ≅150 bases of T-terminated fragments. Even if resolution of larger fragments is poor, this result demonstrates the feasibility of using a thermosensitive polymer for DNA separation.

More recently, Barron's group [23•] synthesised a family of poly(DMA-*S*-DEA) copolymers with different monomer compositions, and demonstrated that resolution decreased with increasing matrix hydrophobicity (i.e. percentage weight of DEA). Indeed, a read length of 421 bases was reached in 75 min with 98.5% accuracy, using a 53% DEA and 47% DMA polymer solution (7.36% w/v, ≅ 4 MDa) [35]. Then, with a less hydrophobic copolymer consisting of 42% DEA and 58% DMA, 575 bases were sequenced in 94 min with 98.5% accuracy (AE Barron *et al.*, personal communication, mentioned in [33•]).

Polyacrylamide grafted with poly(*N*-isopropylacrylamide)

A serious problem with the thermofluidifying polymers described in the previous section, is that the low viscosity state is obtained at a temperature higher than the separation temperature, which is not easy for manipulation, especially for sequencing which requires a separation temperature of 50–60°C. Therefore, polymers with the opposite behaviour (thermothickening) have been proposed.

Copolymers of the thermothickening family consist of a hydrosoluble backbone of PAM grafted with short-chain poly(*N*-isopropylacrylamide) (PNIPAM) to form PAM-g-PNIPAM [36]. PNIPAM is soluble in water below its LCST (≅32°C in water) and at higher temperatures the polymer precipitates. When grafted on the PAM backbone, PNIPAM chains create micelle-like aggregates, which act as transient cross-links and so viscosity dramatically increases (see Figure 3). The transition temperature (T*) is quite different from the LCST of PNIPAM and depends on buffer additives, as shown in Figure 4. The influence of the copolymer's microstructure (i.e. molecular weight, graft size and graft density) on resolution has been studied by separating a 100 bp ladder in denaturant conditions [36]. It appeared that high molecular mass copolymer (2×10^6 Da) with a small graft size (10 000 Da) and a low graft density (≅10% w/v) yielded the best separation resolution. This can be attributed to the decrease of DNA–hydrophobobic microdomain (aggregates) interactions. Using this optimal copolymer and without complete optimisation of electrophoretic parameters (i.e. electic field, concentration and temperature), a resolution of order 0.3 corresponding to the limit of read length with the best performing base calling softwares, could be achieved for 800 bp DNA fragments in less than 1 h.

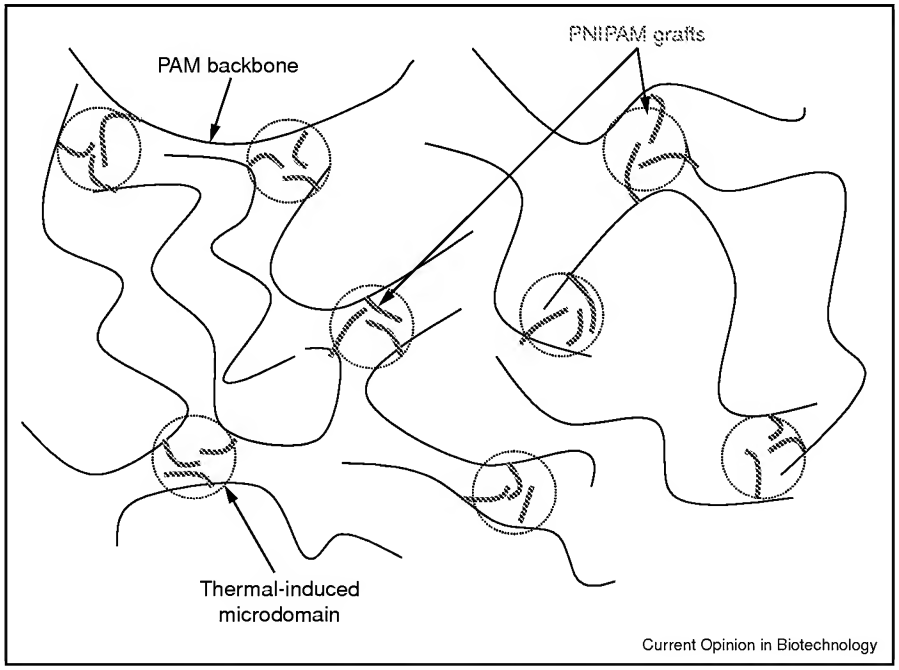
Mixed triblock copolymers: $B_6E_{46}B_6$ and $B_{10}E_{271}B_{10}$

Recently, a mixture of triblock copolymers based on polyoxyethylene (E) and polyoxybutylene (B) has been developed and tested for dsDNA separation [37,38]. In dilute solutions, formation of flower-like micelles occurs, where cores are formed by the hydrophobic B blocks. At higher concentrations, intramicellar links appear and the structure becomes gel-like (see Figure 5). Moreover, gel formation is temperature-dependent, which makes the filling of capillaries easier, and the matrix is self-coating. The resolution is highly sensitive to the blocks length, knowing that B blocks play the major role in the gel-forming process. With pure triblocks, resolution of ΦX174 *Hae*III separation is below that reached with the pluronic F127 ($E_{99}P_{69}E_9$) (where P is polyoxypropylene) [39–41]. On the contrary, results obtained with $B_6E_{46}B_6$ (4–6% w/v) and $B_{10}E_{271}B_{10}$ (3–4% w/v) mixtures are better than with F127, especially concerning the separation of small fragments (single base pair resolution can be achieved up to 100 bp). However, it is difficult to control block-length reproducibility, and mixture formulation has to be optimised each time to reach optimal resolution.

Dynamic polymers of surfactant molecules

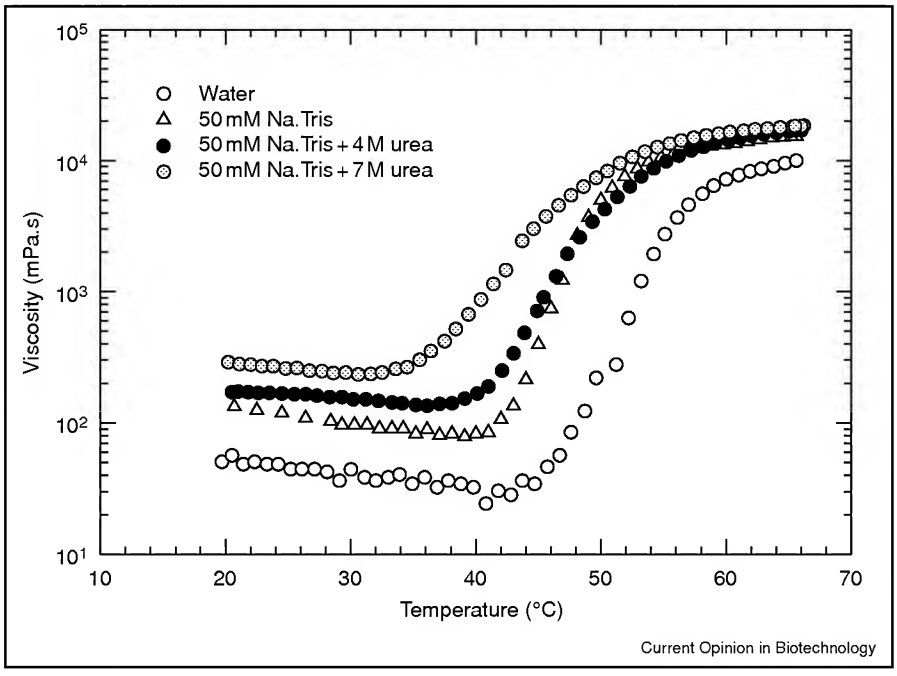
This matrix consists of a hydrophilic polyoxyethylene oligomer (E_8) grafted at one extremity by a C_{16} alkyl chain, which is hydrophobic, resulting in an amphiphilic macromolecule and worm-like micelle formation. With

Figure 3



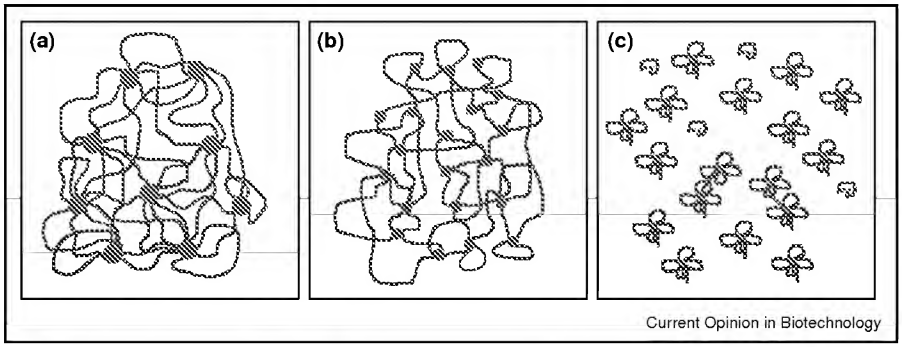
Simplified view of the block copolymer structure above the transition temperature T*. By heating, the LCST grafts undergo a microphase separation and create micelle-like aggregates that act as transient cross-links.

Figure 4



The viscosity of a 3% w/v poly(AM-g-PNIPAM) solution versus temperature. The effect of sequencing buffer additives on the rheological properties are illustrated. For all samples, the shear rate was 10 s^{-1}.

Figure 5



Schematic plots of the structures of **(a)** 8% w/v $B_{10}E_{271}B_{10}$, **(b)** 4% w/v $B_{10}E_{271}B_{10}$ plus 4% w/v $B_6E_{46}B_6$ and **(c)** 8% w/v $B_6E_{46}B_6$.

increasing temperature, micelles get larger and larger and when they begin to entangle with one another DNA separation becomes possible. The effect of temperature has been tested in experiments to separate a 10 bp ladder. Resolution is very poor at low temperature, when micelles are not entangled, and then improves with increasing temperature as the micelles begin to entangle. Above the LCST, phase separation occurs and resolution is lost. This matrix is self-coating and the pore size, which depends on the micelle size, can be controlled by varying the monomer concentration, temperature and the denaturant (influencing the LCST). This should allow the separation of a large range of DNA sizes. So far, DNA sequencing fragments of BigDye G-labelled M13 up to 600 bp have been separated in 1 h [42].

Conclusions

Recently, intensive research has been performed to improve matrices for DNA separation. It is unlikely that a universal matrix will be best for all applications. For sequencing, DNA fragments differing by one base have to be correctly separated up to a large size, and so high sieving performance matrices are required. To our knowledge, commercially available matrices are based on PAM, PDMA and polyoxyethylene. LPA (*CEQ separation gel I*™, Beckman Coulter/*LongRead*® *matrix*, Molecular Dynamics) remains the best performing matrix, but PDMA, which has the advantage of being self-coating and having low viscosity (in contrast to LPA), is also widely used (*POP4*®, *POP5*®, *POP6*®, Applied Biosystems). Many research groups are currently working to develop self-coating matrices that are able to efficiently reduce the electro-osmotic flow, and interactions between DNA and the capillary wall. Low-viscosity matrices are also advantageous as capillary (and more generally microchannel) filling becomes easier and requires a smaller pressure. To this aim, thermoadjustable-viscosity polymers have been developed and the recent ones, such as PAM-g-PNIPAM, $B_6E_{46}B_6$ and $B_{10}E_{271}B_{10}$, are very promising for DNA separation.

None of these new matrices fully equals the best sequencing resolution achieved with LPA in laboratory conditions. Therefore, finding better matrices is still a challenge. In particular a better understanding of the relationships between matrix properties (e.g. hydrophobicity) and performances should help to develop more appropriate polymers. On the practical side, several of the recent wall-coating, low-viscosity matrices have already yielded in the laboratory performance superior to those of the best commercial ones, in identical conditions. Therefore, we can have reasonable hope of seeing some of them commercially available in the near future.

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